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BIOLOGICAL BULLETIN

NUCLEAR CHANGES IN THE REGENERATING SPINAL CORD OF THE TADPOLE OF RANA CLAMITANS.[1]

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I. STATEMENT OF THE PROBLEM.

The present paper gives the results of an histological study of the early stages of regeneration in the spinal cord of the frog tadpole, *Rana clamitans*. It deals especially with the degenerative nuclear changes immediately following the operation, and the phenomena of nuclear division in the formation of the new organ.

Fraisse (1885) studied these stages in several vertebrates in order to discover the origin of the regenerated tissues, and presented the following conclusions which may be used as a basis for a further detailed study.

"1. Sowohl bei Amphibien wie bei Reptilien sind verletzte Gewebe nur im Stande, wiederum gleichartig Gewebe zu erzeugen. Die Leukocyten übernehmen bei der Gewebsbildung nur die

[1] Contribution from the Zoological Laboratory of the University of Illinois, No. 37.

Function der Ernährung; ausserdem nehmen sie zerfallende Gewebsproducte auf und assimiliren dieselben, um sie an anderen Orten wieder zu deponiren. Niemals werden sie selbst zu fixen Gewebszellen, weder in der Bindesubstanz noch sonst wo.

"2. Sämmtliche der in Frage kommenden Gewebe der Amphibien und Reptilien sind im Stande, sich zu regeneriren; entweder direct aus ihren Elementen, oder aus einer Matrix, so lange diese Matrix unverletzt ist. Als Matrix für die Epidermis ist das Rete Malpighii, für das centrale Nervensystem das Epithel des Centralcanales, für die Muskulatur die Muskelkörperchen zu betrachten.

"3. Zuerst regeneriren sich Epithel und Bindegewebe; beides scharf getrennt, ursprünglich aus gleichartigen Zellen bestehend, die sich später differenziren."

There remains the further problem of the stages in the process by which the old organs at the cut surface replace their lost parts. Two distinct kinds of changes take place in this process, (1) degenerative and (2) regenerative. First the injured cells at the cut edge degenerate. Then follows regeneration proper, or the formation of the new organ from the remaining elements of the old.

There are three ways in which regeneration proper might take place. (1) The cells at the cut edge of each organ by dividing might extend outward, and in time form the completed organ: (2) the cells in front of the cut edge might wander backward; and (3) the cells in front of the cut edge might divide in situ and push backward the more distal cells. These possible methods of regeneration will be made clearer by a diagram of that part of the hollow neural tube extending forward from the cut (Fig. 1). If (1) (division of cells at the cut edge) were the method of regeneration, we should find after the operation that

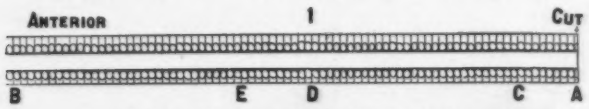

FIG. 1. Diagram, explained in the text.

the cells at the cut surface A, or from A to C, are dividing rapidly while from C to B about the normal number of cells is dividing.

If (2) (migration of cells) were the method, we might find no dividing cells at all, but should expect to find that the cells from *B* to *A* or possibly only from *D* to *A* are turned with their long axes parallel to the longitudinal axis of the spinal cord as if moving toward the cut end. If (3) (division of more anterior cells in situ) were the method, we should expect to find dividing cells all the way from *B* to *C* or possibly concentrated in a growing zone *ED*.

The present paper aims to give an account of the nuclear changes, both degenerative and regenerative, involved in the formation of the regenerated spinal cord.

II. Material and Methods.

Serial sections were made of tadpole tails killed after various regeneration periods. This enables one to follow the process from stage to stage. But to get uniform results from this method and eliminate individual variations, one must take tadpoles as nearly alike as possible at the start, operate on all at the same time, keep them under uniform laboratory conditions and make sections of several individuals at each stage.

On October 12, 1913, seventy tadpoles of *Rana clamitans*, varying in length from 30 to 60 mm., were brought into the laboratory. Two days later they were put into individual finger bowls, and forty-four medium sized individuals (32–40 mm. in length), chosen to constitute the main series, were grouped by twos or threes. Those of each group were as nearly alike as possible and each group was treated as a unit in the time of operation, killing, etc. The finger bowls were placed side by side on a table some distance from the windows so that uniform conditions of temperature, light, etc., were insured. None of the tadpoles was fed during the course of the experiment, and none died from the effects of laboratory conditions.

On October 15, the first operations were performed. Each tadpole was transferred from the finger bowl to a paraffin block and approximately one fourth of the tail was removed, with a sharp scalpel, at right angles to the plane of the tail. The animal was returned to the finger bowl and the removed part put into Gilson's killing fluid. At the end of the period of regeneration, the animals were again taken out onto the block and the

regenerated tail plus a second fourth of the normal tail was removed and put immediately into Gilson's killing fluid. The times of killing were as follows: normal, immediately after the operation, 1, 3, 5½, 9½, and 14 hours, and 1, 2, 3, 4, 6, 8, 9, 10, 12, 14 and 16 days after the operation. Usual methods of technique were followed. Delafield's hæmatoxylin and acid fuchsin stain the nuclei blue and the cytoplasm pink, but do not distinctly bring out cell boundaries. For the most part sections were made in the sagittal plane.

III. Observations.

The study was confined to the histology of regeneration in the spinal cord, since a preliminary examination showed that this organ of all those in the tail was best adapted for a study of the present problem. Fig. 2 shows by a sagittal section the spinal

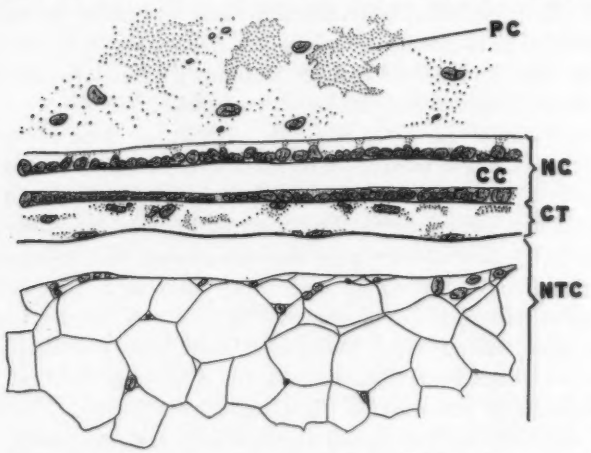

Fig. 2. Sagittal section through a part of the normal tail, showing the spinal cord and its relation to the surrounding tissues. *nc*, spinal cord; *cc*, central canal; *ntc*, notocord; *ct*, connective tissue; *pc*, pigment cell. (330 diameters.)

cord, and its relation to the surrounding tissues. Fig. 3 shows a transverse section of the spinal cord alone. It is a hollow tube which distally is formed of a single layer of cells. The nuclei are very near the inner border of the cells so that there is a wide outer zone of cytoplasm but practically no inner cytoplasmic

zone. At this stage in the development of the tadpole, the cells near the distal end of the spinal cord show little differentiation.

FIG. 3. Transverse section through the normal spinal cord, showing the nuclei and the outer cytoplasmic zone. *cc*, central canal. (890 diameters.)

1. *Degenerative Changes after an Operation.*

When a tadpole's tail is removed the old notocord extends out beyond the other tissues, and the connective tissue between the notocord and spinal cord is usually broken so that the spinal cord bends dorsally as in Figs. 7 and 8. A transverse cut through the tail leaves the various organs at the cut surface in contact with the surrounding medium, the water in which the tadpole lives. Sections of tadpoles killed immediately after the operation show the direct effect of the cutting (Figs. 4 and 5). Many

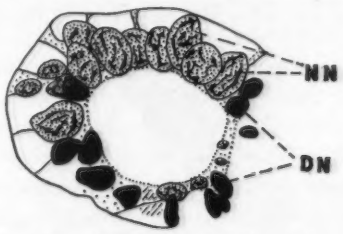

FIG. 4. Transverse section through the end of the spinal cord immediately after the operation, showing deeply-staining nuclei. *cc*, central canal; *nn*, normal nuclei; *dn*, deeply-staining nuclei. (920 diameters.)

nuclei and cells are broken and irregular in appearance and may be loosened or torn apart from each other. The injured nuclei at the cut edge and extending forward with decreasing frequency, are homogeneous in appearance and take a deep haematoxylin stain. Undoubtedly some of the nuclei are cut, and this accounts for the irregularity in shape of a good many. But a good many others, also staining deeply, are rounded and smaller than normal nuclei. These may be either normal nuclei which under the

stimulus of the operation are contracted or compressed, or cut nuclei which have rounded off. These deeply-staining nuclei, whether rounded or irregular in shape, are smaller than normal

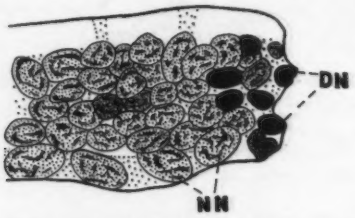

FIG. 5. Sagittal section through the side of the spinal cord immediately after the operation, showing the deeply-staining nuclei at the cut end. *dn*, deeply-staining nuclei. (920 diameters.)

nuclei, so it may be that the chromatin, which stains deeply, is condensed on account of the loss of achromatic material.

The same assumption is borne out by the somewhat different appearance of nuclei in the tadpoles killed one hour after the operation (Fig. 6). Some are rounded as before; others are angular or slightly hour-glass shaped, with rather dense cytoplasm extending out from the corners. If parts of the nuclear

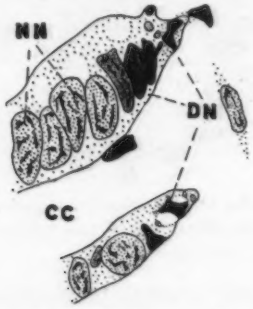

FIG. 6. Sagittal section through the spinal cord one hour after the operation. This shows the "contracting" nuclei. *cc*, central canal; *dn*, deeply-staining nuclei; *nn*, normal nuclei. (920 diameters.)

membrane were held by the cytoplasm while the nucleus as a whole decreases in volume either by contraction or loss of achromatin, the nuclei might present such an appearance. Moreover there are gradations from hour-glass-shaped to normal nuclei

and corresponding gradations in size and depth of stain. In cases of this sort there are often vacuoles or cytoplasm between the nuclei as if the latter had shrunken, whereas in the normal cord, the nuclei are so close together that no cytoplasm can be seen between them. These facts indicate that normal nuclei become deeply staining nuclei by contraction or by loss of achromatic material.

This "contraction" of nuclei seems to be caused by contact with the water or killing fluid, or the succession of the two, as well as by direct injury from the scalpel, for other nuclei which are in contact with the exterior only through the central canal show this phenomenon. In some cases, the end of a nucleus nearest the central canal is deeply stained and contracted while the other part is normal (Fig. 4). The question immediately arises, why does not the water or other external factor enter the open neural tube and cause the contraction of the inner parts of practically all nuclei in the spinal cord? It is probably because of the presence in the tube of some substance which prevents the ready admission of external fluids, though capillarity would have a similar effect. Since the sections show very little structure within the central canal, this content must be liquid or semi-liquid. However, in a number of sections there is a rather long narrow band of cytoplasmic material which may be the more solid part of a semi-liquid substance coagulated by the killing reagent. There are other evidences of the presence of such a liquid. The sections from two of the tadpoles killed one hour after the operation show a coagulation of the outer surface of the blood plasma covering the wound, but over the spinal cord this coagulating process is delayed. The most plausible explanation seems to be that some cerebro-spinal fluid (compared by Barfurth to the cerebrospinal fluid of mammals) exerts an outward pressure which breaks through any slight hardening of the plasma at this point. Perhaps transference of the animal to a medium of different density, the killing fluid, aids the outburst. Sections of another tadpole killed at one hour show the presence of this coagulated plasma over the end of the spinal cord as well as over other parts of the tail.

The outward pressure of a fluid would tend to push out into

the blood plasma any free elements such as the injured and degenerating nuclei with very little cytoplasm and hence little connection with other cells; and when this fluid breaks through, some of these nuclei may break off and float away. At one hour after the operation, broken and small rounded nuclei are seen in

FIG. 7. Sagittal section through the spinal cord and the surrounding region one hour after the operation, showing irregularly shaped, deeply-staining nuclei in the end of the spinal cord and in the coagulated plasma layer. *dn*, deeply-staining nuclei; *pl*, plasma layer; *ct*, connective tissue; *ntc*, notocord. (1,100 diameters.)

the end of the spinal cord and extending out into the hardened layer of the plasma, giving evidence of some force acting outward at this time (Fig. 7). Other evidences will be mentioned in describing the stages at which they appear.

Three hours after the operation there are fewer of the angular nuclei than at one hour and more of the round deeply-staining nuclei. The latter vary from the size of similar ones in the earlier stages down to fragments. Moreover some of the larger of these seem to be in the process of fragmentation, that is, appearances indicating stages in direct division are seen. The gradation in size and depth of stain at one hour from normal nuclei nearly to rounded ones, and the gradation down to fragments at three hours, as well as the appearances of fragmentation, make it fairly clear that normal nuclei just in front of the cut edge may contract, become rounded, and fragment. This must be a degenerative process. Even finer intermediate steps are seen in preparations of later stages.

Sections of one individual at this period appear very much like those immediately after the operation. The deeply-stained nuclei are similar, and the spinal cord is not covered either by epidermis or plasma, so that a recent outbreak of the cerebrospinal fluid must have taken place. In this case a second contact with the exterior has again started the degenerative process.

At five and a half hours the spinal cord is entirely covered by the thickened plasma layer, in which is a group of fragmenting globular nuclei. In one preparation at this time, the epidermis has closed-in over the entire wound, and there is a series of stages in the degeneration of nuclei. Some are only slightly smaller and darker than normal nuclei; others have the angular appearance characteristic of nuclei one hour after the operation, while still others are round and fragmenting. At this stage there is another evidence of the presence of a cerebrospinal fluid. The plasma covering the end of the spinal cord is pushed outward, making a knob-like extension of the central canal similar to that shown in Fig. 8. This did not appear in earlier stages either because not enough cerebrospinal fluid was present, or because the plasma layer had not coagulated sufficiently to resist the outward pressure of this fluid.

Of the two preparations of tadpoles killed after a nine and a half hour interval, one shows the epidermis and plasma covering all the wound except the neural tube; the other shows this part also covered. In the former, the sides of the neural tube are

separated as if by a recent outburst of cerebrospinal fluid, and deeply-staining rounded and fragmenting nuclei are seen. In the second preparation, the deeply-staining nuclei are all small

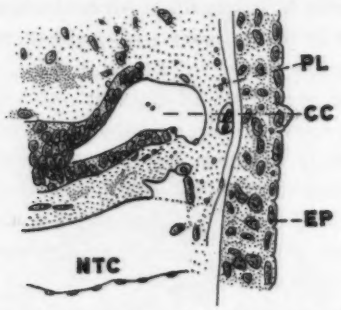

FIG. 8. Sagittal section through the end of the spinal cord fourteen hours after the operation. This shows the epidermal layer, the plasma layer, and the knob-like extension of the central canal, caused by the outward pressure of the cerebro-spinal fluid. *ep*, epidermis; *cc*, central canal; *pl*, plasma layer; *ntc*, notocord. (330 diameters.)

and fragmentary. In other words no more nuclei seem to be starting to degenerate.

At fourteen hours, plasma and epidermis cover the spinal cord though the plasma is pushed outward by the cerebrospinal fluid (Fig. 8). There are nuclear fragments in the cord and degenerating nuclei in the plasma. Another preparation of the same period shows the nerve cord still open to the exterior, as well as the nuclear appearance of an earlier stage.

At twenty-four hours, only a few of the nuclei are slightly smaller and darker than the normal. At this time there appear near the end of the spinal cord, granular leucocytes containing pigment granules and fragments which closely resemble the fragments of degenerating nuclei. It may be that the leucocytes appear at this time and dispose of nuclear fragments. After one day, the degenerating nuclei are too rare to be significant.

The degenerative process which the foregoing facts seem to show, may be indicated diagrammatically as follows:

Cells directly cut → broken nuclei → rounded nuclei → fragments → disposed of by outbreak of cerebro-spinal fluid, or by leucocytes.
Cells just in front of those cut → angular nuclei → rounded nuclei → fragments → disposed of by leucocytes.

2. *Enlargement of Nuclei.*

A few preparations of the spinal cord soon after the operation show plainly that the nuclei near the end, but just in front of the deeply-staining nuclei, are larger than those of the normal cord. The long axes of nuclei close to the edge were measured and compared to nuclei of the same preparation which are some distance forward in the old tissue (Table I.). Immediately after the

TABLE I.

Time of Regeneration.	Nuclear Length Close to Edge.	Nuclear Length in Front of Edge.	Difference in Length.
Normal	7.9	7.5	.4
Immediately	10.5	7.8	2.7
1 hour	11.3	9.1	2.2
3 hours	8.6	7.3	1.3
5.5 hours	12.8	10.5	2.3
9.5 hours	8.1	8.0	.1
14 hours	7.6	8.3	−.7
1 day	8.2	6.6	1.6
2 days	8.1	8.0	.1
3 days	8.2	8.9	−.7
4 days	8.4	8.2	.2
6 days	10.7	10.4	.3

Explanation.—Each measurement recorded here is the average of the measurements of 9 or 10 nuclei. These were recorded in terms of the spaces of the ocular micrometer, but since one space was equal to approximately one micron (.955), the measurements were not transposed.

operation and in the very early regeneration stages, the nuclei near the end are larger, but the difference decreases until after nine and a half hours it is hardly significant. This enlargement might be preparatory to normal division or it might be a swelling which is a degenerative change preliminary to fragmentation Since this size difference is greatest at the very beginning and decreases during the first day until it is no longer significant, and since mitotic divisions are not seen in numbers until the third day, the enlargement is probably an early stage in nuclear degeneration.

3. *Temporary Partial Closing of the Spinal Cord.*

After the degenerative process is complete and the deeply-staining nuclei have disappeared, the end of the nerve cord starts to close over. By the first day, the nuclei in the end of the cord

have begun to pull apart, stretching out the connecting cytoplasm (Fig. 11). In general they extend toward the opposite

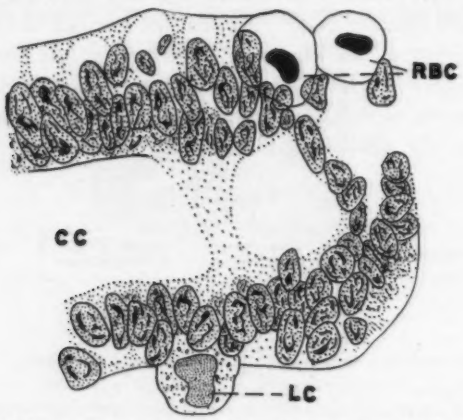

FIG. 9. Sagittal section close to the edge of the central canal, showing a row of cells, not quite at the end, extending across the central canal. Other sections of the series show that the end of the cord is still open. *cc*, central canal; *rbc*, red blood corpuscles; *lc*, leucocyte. (920 diameters.)

wall of the central canal, thus narrowing the opening at the end. Some sections show pseudopod-like cytoplasmic extensions of the cells into the central canal as if closing were to be produced

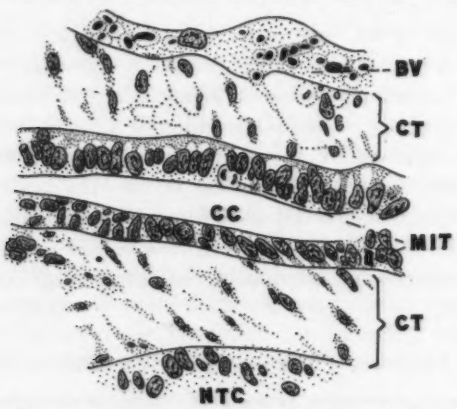

FIG. 10. Sagittal section through the new spinal cord six days after the operation. *bv*, blood vessel; *mit*, mitotic figures; *ntc*, notocord; *ct*, connective tissue. (330 diameters.)

by amoeboid movement of the cells. Figure 9 shows a section through one side of the cord, in which one layer of cells, not quite at the end, is extending down into the central canal. Up to about six days phenomena such as these may be seen, but sections from six to sixteen days show that the closing is not completed within that period. By sixteen days the new tail is

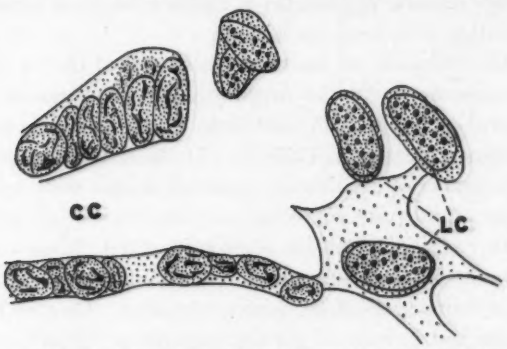

FIG. 11. Sagittal section through the spinal cord one day after the operation showing the granular leucocytes at the end of the cord, and the pulling apart of nuclei in the lower part of the cord. *cc*, central canal; *lc*, leucocytes. (920 diameters.)

almost as long as it will become (Durbin, 1909), and the spinal cord reaches back close to the epidermis at the posterior end. Still these later preparations show the sides of the neural tube gaping open, and red blood corpuscles extending forward into the central canal of the new cord, as if the pressure of the cerebrospinal fluid is not sufficient to keep them out.

4. *Cell Division.*

In an organ such as the spinal cord in which the nuclei lie close together, it is difficult to determine an amitotic division. In order to be sure that amitotic divisions do occur, one must find continuous stages in nuclear and cellular constriction without the formation of chromosomes. Because of the massing of nuclei, this cannot readily be determined in the normal spinal cord, though the slides were examined with this point in mind. The present study gives no evidence that normal nuclei divide amitotically, but stages in direct division can be seen in the

deeply-staining nuclei at the cut edge. Is this amitosis or fragmentation? Do the daughter nuclei form normal nuclei, or do they divide several times and degenerate? There is no definite evidence that nuclei which divide directly ever become normal again. But at successive stages the deeply-staining nuclei become smaller and smaller down to fragments, so that the direct division is probably a fragmentation as a part of the degeneration of injured nuclei.

Mitotic divisions can easily be distinguished by the formation of chromosomes. All the preparations were examined and the distance of each mitotic division from the cut edge was measured. The results are shown in Table II. In the sections of the normal tail the number of divisions is the smallest, but since up to three days the mitoses are scattered and the number of individuals small, there is no reason for considering these mitoses anything but normal. During the period of degenerating nuclei, there are almost no mitotic divisions close to the edge. On the third day, the nuclei just in front of the cut edge are proliferating rapidly; at four days there are a few divisions past the cut; at six days there are almost as many divisions in the new spinal cord as in the old; at eight and nine days most of the divisions are in the new cord; at fourteen days there are scattered mitoses only, both in the old and new cord, and at sixteen days most of the dividing cells are in the very end of the new cord. If later divisions follow this general trend, it seems likely that the rest of the spinal cord will be formed by a growing zone at the tip, and until the new cord is complete the number of mitoses near the tip would probably decrease gradually.

Fig. 12 gives the average number of mitoses in the spinal cord at each stage, and therefore represents the rate of growth at these times. On the second day there is a considerable mass of tissue over the whole wound, though only degenerative changes have been taking place in the nerve cord. Beginning about this time, the nuclei in the end of the cord loosen and draw apart somewhat, stretching out the cytoplasm between them (Fig. 11). This is apparently the first extension in length of the spinal cord. At three days active proliferation of cells has begun but the pulling apart or stretching toward the cut edge continues. Fig.

TABLE II.

Regeneration Time.	No. of Individuals.	Mitoses in Old Cord.	Mitoses in New Cord.	2.8 mm.	2.1 mm.	1.4 mm.	.7 mm. ← Old Tissue.	Position of Cut.	New Tissue. → .7 mm.	1.4 mm.
Normal..	3	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$	0		$\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$				
Immed...	3	2	0	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$			$\frac{1}{2}$			
1 hr.	3	6	0	$\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}$ 1 $\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}\frac{1}{2}\frac{1}{2}$	$\frac{1}{2}$ $\frac{1}{2}\frac{1}{2}\frac{1}{2}$			
3 hrs.....	2	$1\frac{1}{2}$	0	1		$\frac{1}{2}$ $\frac{1}{2}$				
$5\frac{1}{2}$ hrs....	2	$2\frac{1}{2}$	0	1 $\frac{1}{2}$			$\frac{1}{2}$			
$9\frac{1}{2}$ hrs....	2	$1\frac{1}{2}$	0		$\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$	$\frac{1}{2}$				
14 hrs....	2	$3\frac{1}{2}$	0	$\frac{1}{2}$		1 $\frac{1}{2}$	$\frac{1}{2}$			
1 day....	3	$1\frac{1}{2}$	0		$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$ $\frac{1}{2}$			
2 days...	4	$1\frac{1}{2}$	0		$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$ $\frac{1}{2}$			
3 days...	1	19	0				6 4 8 1			
4 days...	2	15	$2\frac{1}{2}$	$\frac{1}{2}$ $\frac{1}{2}$ 1	$\frac{1}{2}$	$\frac{1}{2}$	$\frac{1}{2}$ 3 $2\frac{1}{2}$ 6		1 $1\frac{1}{2}$	
6 days...	2	$19\frac{1}{2}$	19	$\frac{1}{2}$ $\frac{1}{2}$ $\frac{1}{2}$		1 1 $\frac{1}{2}$ 2	$2\frac{1}{2}$ 2 $2\frac{1}{2}$ 4 $2\frac{1}{2}$		4 $3\frac{1}{2}$ $2\frac{1}{2}$ $6\frac{1}{2}$ $2\frac{1}{2}$	
8 days...	1	2	40				1 1		3 12 6 5 4	1 3 6
9 days...	1	16	42			1 1 1 3	3 1 6		2 7 4 5	3 3 4 9 5
14 days..	2	6	6		$\frac{1}{2}$	$\frac{1}{2}$ $\frac{1}{2}$ $1\frac{1}{2}$	$1\frac{1}{2}$ 1 $\frac{1}{2}$		1 1 $\frac{1}{2}$ 1	$\frac{1}{2}$ 1 1
16 days..	2	$\frac{1}{2}$	$7\frac{1}{2}$			$\frac{1}{2}$			1 $\frac{1}{2}$	3 3

This table represents the position and number of mitotic divisions taking place at different times. In most cases the number given is the average obtained from 2 to 4 individuals, as indicated in the table.

10 (six days' regeneration) shows the cells in one part of the cord stretched out to such an extent that vacuoles are left between the

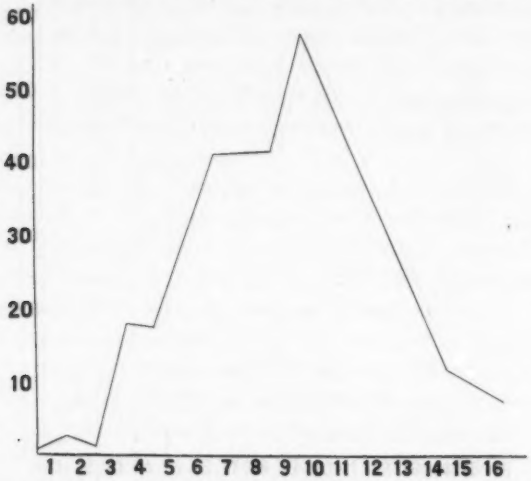

FIG. 12. Curve giving the number of mitotic divisions in that part of the spinal cord within 3 mm. of the edge. Beyond 3 mm. the mitoses are scattered. An abscissa represents the period of regeneration and the corresponding ordinate gives the average number of mitoses found in the individuals killed at the end of that period.

cells. It is during the period from four to sixteen days that most of the increase in length takes place, by active proliferation and migration of cells.

IV. DISCUSSION.

1. *Amitosis and Fragmentation.*

Fraisse in describing the regulative process at about two days after the operation, says; "Bereits früher machte ich darauf aufmerksam, dass am Wundrande eine starke Auswanderung von Leukocyten stattfindet, und dass diese es sind, welchen vor allen Dingen die Bildung des homogenen, lymphartigen Saumes, welcher zuerst die wunde bedeckt, zuzuschreiben ist. Das Rückenmark geht nun an meinen Schnitten bis dichte an diesen homogenen Saum heran, und die Elemente, welche es zusammensetzen, lassen sich immerhin noch nach 24 Stunden auch an

diesem Saum von einander trennen, dann aber tritt eine bedeutende Wucherung von Kernen auf, und zwar scheint dieselbe auszugehen von den sogenannten Körnern,[1] deren Inhalt völlig homogen und stark lichtbrechend erscheint. Durch Picrocarmin werden diese Elemente ebenfalls stark tingirt, und nun sieht man an diesen nahezu gleich grossen Körnern Kerntheilungen, ohne dass jemals eine Spur von karyokinetischen Figuren constatirt werden konnte, in der Weise auftreten, das der Kern oder die Körner sich in der bekannten Weise schuhsohlenförmig einschnüren, und dass dann aus beiden Hälften Elemente gleicher Art hervorgehen. Nicht nur eine einmalige Einschnürung glaube ich beobachten zu können, sondern auch eine mehrfache, so dass der Kern sich bei diesem Process nicht nur in zwei, sondern auch in mehrere Stücke theilen kann."

Fraisse discusses further the evidence that the nuclei from the end of the spinal cord, which are found in the lymph-like border, divide amitotically. This agrees with the present observations. But he is satisfied to show that direct division does take place. So far as my preparations show, there are few evidences that the nuclei which divide amitotically afterward become normal nuclei. In some of the preparations of stages at which the deeply-staining nuclei have almost disappeared, there are a few nuclei which stain only slightly darker than the normal ones, and at this time there are no stages between these and the fragments. These few slightly darkened nuclei may, then, be forming normal nuclei again. All other evidence points towards the conclusion that at successive stages, these deeply-staining nuclei become smaller and smaller as if fragmentation or repeated direct division, is taking place. The conclusion from these facts is that nuclei which have only started to degenerate may perhaps return to the normal condition, but that nuclei that have gone so far as to divide amitotically are destined to fragment.

2. *The Appearance of Leucocytes.*

Barfurth (1891), working on the regenerating spinal cord of the frog larva at forty-six hours and at three days, makes the

[1] Körnern-nuclei of the gray substance, which are not present in the distal region of the spinal cord.

following statement: "Die unterste Theil des regenerirten Medullarrohres beherbergt in seinem Innern und zwischen seinen Epithelzellen zahlreiche fettig degenerirende Leukocyten; viele kleine und grosse Fetttropfen, die man hier überall findet, führe ich ihrem Ursprunge nach auf solche zerfallene Wanderzellen zurück. Ausserdem finden sich hier auch viele Pigmentkörnchen, die wohl bei der regressiven Metamorphose der zerfallenden Leukocyten entstehen (Pigmententartung)."

Barfurth figures the spinal cord of a larva of *Triton cristatus* after the sixth day of regeneration, in which these leucocytes and fat drops are shown. His figure is very similar to Fig. 11, which shows a section of a tadpole killed twenty-four hours after the operation. Both Fraisse and Barfurth mention particularly the presence of leucocytes in the early regeneration stages, but in the present study, leucocytes were not found in large numbers. Up to the end of the first day, none at all were seen close to the spinal cord. The earliest stage mentioned by Barfurth is that after a forty-six hour regeneration period, and this probably accounts for the different interpretation he gives of the origin of the "Fetttropfen" or fragments. If these fragments are followed back into earlier stages in my sections, they become larger and larger and are seen to be identical with the degenerating nuclei. To be sure, the leucocytes when they first appear in the spinal cord region contain what might be called fat drops, but is it not more reasonable to suppose that the leucocytes which are present at this time dispose of the fragments of injured spinal cord nuclei?

3. *Temporary Closing of the Spinal Cord.*

Barfurth describes the closing of the spinal cord at three days by means of cytoplasmic extensions of the cells, such as were seen in the preparations used in the present study. "Der sich wieder ansammelnde Liquor cerebrospinalis drückt nun auf die neugebildeten, noch wenig resistenten untern und seitlichen Theile des Rohres, und treibt sie kolbenartig auseinander. Die Zellen passen sich einstweilen durch ihre Lagerung diesem Druck an und behalten später diese Lage noch eine Zeit lang bei." Barfurth mentions this as a temporary closure of the spinal cord, so

his later preparations evidently show the cord again open. The regenerated spinal cord at sixteen days has almost reached its maximum length, but it is not yet closed. Whether or not the completely regenerated spinal cord is open at the end or closed as in the normal tail cannot be answered by the present study.

4. *Rate of Division. Amitosis versus Mitosis.*

Durbin (1909), in analyzing the rate of increase in length throughout the regenerative process in the tail of *Rana clamitans*, distinguishes four periods. "The operation was followed by an interval of low rate, succeeded by one of rapidly increasing rate, then by one of rapidly decreasing rate and finally an interval in which the rate gradually approaches zero. The first low period is explained by a combination of two factors, (*a*) the shock of the injury, and (*b*) the formation of a cap of embryonic cells which is to serve as a basis for the more active regeneration. The second or period of rapidly increasing growth is the one in which practically all the cells in the new part are undifferentiated and rapidly dividing. The third and fourth periods are explained by the appearance of differentiation, which lessens the number of dividing cells."

Fig. 12, based on the number of mitotic divisions in the spinal cord, shows these same periods. The initial period of low rate covers the first two days; that of rapidly increasing rate includes the third to ninth days; the period of rapidly decreasing rate extends from the tenth to sixteenth days, and the period of gradually decreasing rate, though not covered in the present work, would undoubtedly extend on from about sixteen days. In the light of this histological study, a somewhat different interpretation might be given to the initial period. It is during these first two days that degeneration of the injured cells is taking place. Though at this time a cap of undifferentiated cells is being formed over the wound, the spinal cord does not participate in the formation of this cap, nor is any such cap formed at the end of the spinal cord. Since the spinal cord cells in this part of the tail are so slightly differentiated, the new cord is formed from the old without the separation of a group of special embryonic cells.

The similarity of the rate curves based on a counting of the mitotic divisions with that based on the amount of tissue formed at each period, seems to be significant. It shows that the rate of tissue formation is closely correlated with the number of mitotic divisions. Considering amitosis, this may be interpreted in one of two ways—(1) either the number of amitotic divisions is similarly correlated with the rate of growth so that the total number of divisions both mitotic and amitotic, gives the same form of curve as the mitotic divisions alone, or else (2) amitotic divisions are not numerous enough to be significant. The former explanation is improbable. The nuclear conditions producing mitotic division are probably different from those producing amitotic division. Different cells in the same region may divide by different methods, but it is very improbable that the conditions producing one form of division would increase and decrease in influence at the same rate and the same times as those producing the other form. Moreover, in the present study, no examples of direct division were seen except in the degenerating, fragmenting nuclei. This similarity of the rate curve of mitotic divisions to the rate curve of growth is evidence, other than the negative observational evidence, supporting the view that amitotic division is not important in the formation of this organ by regeneration.

V. Summary.

1. The regenerating spinal cord of the frog tadpole has been studied histologically in order to learn the mechanism, or the stages in the process, by which the new cord is formed from the old.

2. During the first day after the operation, injured nuclei in the end of the spinal cord degenerate. There is first a decrease in size, by contraction or loss of achromatin, and then a fragmentation of these degenerating nuclei. The fragments may be carried away either by the outbreaking of a cerebrospinal fluid or by leucocytes which appear at this time. These fragments are parts of disintegrated spinal cord nuclei and not of leucocytes.

3. From the second to the sixth days there is a temporary partial closing of the neural tube, probably by migration of the cells near the end.

4. The new cord is formed by the cells of the old cord near the cut edge, by mitotic division and migration.

5. The number of mitotic divisions at different periods is proportional to the rate of regeneration at those periods as determined by Durbin. Amitotic division, if it occurs, is not important in the formation of the regenerated organ.

6. There is no observational evidence from this study that amitotic division does occur in normal regenerating spinal cord cells.

This work was carried on under the direction of Dr. Charles Zeleny. His suggestion of the problem, and constant interest in its progress are sincerely appreciated.

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NOTE ON THE EFFECT OF X-RADIATION ON FERTILIZIN.[1]

A. RICHARDS AND A. E. WOODWARD.

The observations of one of the writers that x-rays would produce changes in the activity of certain enzymes suggested that these rays might perhaps be effective in bringing about changes in the action of cell extractives, particularly of fertilizin, the descriptions of which by Lillie and Glaser appeared at the beginning of the summer. The opportunity was presented to test this suggestion during the past summer at Woods Hole, since the one of us carried on studies on the effect of x-rays on some marine eggs and the other continued the work on fertilizin of *Arbacia* begun by Glaser. This note gives a summary of the results. It is realized by the writers that the study is by no means a complete one, but it is believed by them that publication is justified in view of the facts that the experiments give clear evidence on the main point under investigation and that there is at present no prospect of opportunity for their further work on the problem.

In taking up these experiments the writers felt that if it should be shown that x-radiation influences the activity of the cell extractive called fertilizin, that fact would be of interest from several view points: (1) without regard to the nature of fertilizin or its rôle in the fertilization of the egg, it is a substance derived from the eggs which has the property of being definitely modified by those external agents of which experimental use may be made; (2) in cell extractives, of which fertilizin is an example, there is a basis for the action of x-rays upon living cells, and doubtless the marked effects of the rays upon tissues is partially due to such action; (3) the modifiability of its activity by radiation is an interesting property of fertilizin; (4) this property may serve as a point in determining the relation of fertilizin to enzymes.

[1] Contribution from the Marine Biological Laboratory at Woods Hole, and from the Zoology departments of the University of Texas (No. 123) and of the University of Michigan.

The methods used in these experiments were largely those of Lillie and Glaser. The solution used as a standard was prepared according to the method of Lillie by "adding to a certain number of 'dry' eggs, double their volume of sea-water, and with occasional slight agitation allowing ten minutes to elapse. At the end of this time the ova were precipitated by 100 revolutions of the centrifuge and the supernatant fluid, a clear, golden liquid in the case of *Arbacia*," was decanted, (Glaser, 14*a*). The agglutination of fresh sperm in suspension by fertilizin in both control and radiated solutions, was tested by the unit concentration method of Lillie, of which he says, (13*b*) "The agglutination reaction of the sperm in the presence of this substance (*Arbacia* fertilizin) is, as noted in previous studies, reversible, and the intensity and duration of the reaction is a factor of the concentration of the substance. The entire reaction is so characteristic that it was possible to arrive at a unit by noting the dilution at which the least unmistakable reaction was given. This was fixed at about a five or six-second reaction, which is counted from the time that agglutination becomes visible under a magnification of about 40 diameters until its complete reversal. The unit is so chosen that a half dilution gives no agglutination of a fresh 1 per cent sperm suspension." Further details are given in his recent paper ('14, pp. 526–528). One can best observe the details of the reaction with the low power of the microscope. The sperm suspension is mounted under a cover glass and the drop of fertilizin added at the edge of the suspension by means of a pipette. The entire process is observed through the microscope, and the time elapsing before the complete reversal of the reaction is carefully noted by means of a stop-watch. Thus it was possible to determine the degree of activity of a given sample of fertilizin, and by comparing radiated and non-radiated solutions, to measure the effect of the radiation by x-rays.

Another possible method of studying the effect is suggested by the fact that fertilizin can be used to bring about the parthenogenetic development of *Arbacia* eggs, the so-called auto-parthenogenesis. The efficacy of fertilizin before and after radiation in bringing about auto-parthenogenesis is a measure of the action of the radiation on it.

In all the experiments with sperm it has been our policy to use only data from clear cut reactions in which the beginning and the end of the agglutination were definitely marked. Precautions were taken to see that the sperm suspension was fresh and clean. Lillie has shown that both of these factors are important, for an old suspension becomes inactive and the presence of impurities such as blood acts as an inhibitor of the reaction.

Previous experience (Richards, 14) has shown the radiations to be of three kinds in relation to their effect on enzymes depending on duration, intensity and distance of the object from the x-ray tube; namely, accelerative, non-effective, and inhibitive. Under the conditions which usually prevailed in these experiments, a short exposure, of about 2 minutes, is accelerative; an exposure of about five minutes is non-effective; and one of longer duration becomes inhibitive. In view of these facts, similar exposures of fertilizin were made and the resulting activity tested as already explained.

In a preliminary experiment on July 8 the following figures were obtained as the average of a number of readings of the time elapsing before the complete reversal of the agglutination reaction after short and long radiation of fertilizin. The fertilizin solution used was about 2 per cent. standard strength (in this early experiment the strength was not accurately determined, but it is not strictly necessary under the conditions of this test that it should be known exactly). For the control, non-radiated solution the average reaction time was 32 seconds; for the 2-minute radiation the average time was 33 seconds; and for the 15-minute minute radiation it was 23½ seconds. This solution was then diluted to one-half and these figures obtained: Control, 19 seconds; 2-min. radiation, 20 seconds; 15-min. radiation, 16 seconds. This experiment is incomplete and the differences lie nearly within the limits of variation, but they suggest definitely that the short radiation rendered the fertilizin slightly more active (that is, enabled it to hold the sperm in agglutination longer), and the long radiation caused it to be less active than the control. More decisive data would have been given had the dilutions been continued to unit concentration, a fact which led to the adoption of that method in subsequent experiments.

In another experiment (July 14) a 1/50 dilution (2 per cent. standard) of *Arbacia* fertilizin was used. It was separated into four parts, of which one (Sc) was kept as a control solution, one (S2) was radiated 2 minutes, one (S5) five minutes, and the last (S7) seven and a half minutes. The results of these solutions when tested for their agglutination time at successive dilutions to unit concentration are given in the following table. ½Sc means control solution diluted to one-half; ¼ Sc, diluted to one-fourth, etc. The difference between two successive reaction times is marked *d*. Unit concentration is indicated by the asterisk(*).

TABLE I.

Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.	Successive Dilutions.	Reaction Time.	Value of *d*.
Sc.	34 sec.		S2	37 sec.		S5	34 sec.		S7	29 sec.	
½ Sc.	22 sec.	12	½ S2	23 sec.	14	½ S5	27 sec.	7	½ S7	22 sec.	7
¼ Sc.	17 sec.	5	¼ S2	15 sec.	8	¼ S5	19 sec.	8	¼ S7	17 sec.	5
⅛ Sc.	10 sec.	7	⅛ S2	11 sec.	4	⅛ S5	12 sec.	7	⅛ S7	12 sec.	5
1/16 Sc.	4–5 sec.*	5	1/16 S2	7 sec.	4	1/16 S5	5 sec.*	7	1/16 S7	8 sec.*	4
			1/32 S2	4 sec.*	3				1/32 S7	0 sec.	

Inspection of this table shows that the activity of S2 was increased by the short radiation, for five dilutions were required to reduce it to unit concentration, whereas that state was reached in four dilutions in the other three solutions; also the full strength of this solution held the sperm in agglutination longer than did that of the control, 37 against 34 seconds. In other words, Sc was 800 units agglutinating strength, S2 was 1,600 units, S5 and S7 were each a little over 800 units, and much below 1,600 units strength. (Lillie, '14, p. 527.)

The number of dilutions required in S5 was the same as in Sc and the sperm were agglutinated the same time by both solutions. This is in line with the previous experience that a radiation of about five minutes' duration under the conditions of these experiments is non-effective. However, these figures give an additional fact of possible significance which has not been entirely confirmed by other experiments either on fertilizin or on enzymes such as pepsin. If *d* represents the differences between the number of seconds required for the reversal of the reaction by successive dilutions, its value in S5 is practically a constant, 7; but in Sc and

S2 it begins as a large number and decreases rapidly: in Sc its successive values are 12, 5, 7 and 5, while for S2 they are 14, 8, 4 and 4. In S7 the values of *d* are smaller and decrease more slowly, being 7, 5, 5 and 4. This suggests that the laws governing the agglutination reactions by the various solutions are of different character. But in as much as this interesting result has not been generally obtained it is not possible to attach special importance to it at this time. It is given merely as suggestive.

The data in the case of S7 indicate that the activity of the fertilizin was decreased although the number of dilutions was the same as in the control, because the number of seconds required for the reversal of the reaction at unit concentration was much larger than is usual; yet at a further dilution no reaction was obtained. Also the undiluted solution did not hold the sperm in agglutination as long as in the control. Furthermore, it may be significant that the value of *d* for S7, as indicated above, are smaller than in the case of the other solutions.

Subsequent experiments along the same line gave similar results. They show clearly that radiation by x-rays is capable of changing the activity of fertilizin, and in general agree with previous work that weak radiation is accelerative and strong inhibitive. Some of our experiments were performed during the latter part of the summer at the end of the breeding season and there were irregularities in the results, but it is believed that these irregularities may be attributed to the unsatisfactory condition of both sperm and eggs at this season of the year and that the statement above gives the true effect of radiation on fertilizin.

Also during the latter part of the summer the writers tested the effect of x-radiation on fertilizin with regard to its power of inducing auto-parthenogenesis. Due to the near end of the breeding season these results are not entirely trustworthy, but they agree fully on one point, namely, that the radiation effects changes in the capacity of fertilizin to induce parthenogenesis.

On August 10 a sperm agglutination experiment was performed which possibly throws some light on the irregularity of the auto-parthenogenesis and at the same time makes the auto-parthenogenesis test doubtfully applicable for the radiation problem. This experiment gave data showing that the radiation effects

wore off when the fertilizin had stood for some time. If this is true in general it must follow that, since the fertilizin must stand in the parthenogenesis experiments, there would be irregularity in the results.

The only tests of the effect of x-radiation on *Asterias* fertilizin were made on July 28, when the fertilizin was divided into four portions, as usual. One was kept for a control, one radiated two minutes, one five minutes, and the fourth fifteen minutes. The fertilizin was then put on mature *Asterias* eggs, which were allowed to stand two hours in the solution. They were then rinsed with sea-water and treated with hypertonic sea-water (50 c.c. sea water + 8 c.c. 2.5 M. NaCl) for thirty minutes, washed again with sea water, and allowed to stand for 12 hours. All four lots of eggs showed parthenogenetic development, and those treated with fertilizin which had been radiated 2 minutes had a much larger percentage of cleavages than either the control or the others.

Several times *Arbacia* fertilizin was similarly subjected to x-rays and then tested for its auto-parthenogenetic effect on fresh *Arbacia* eggs. The experiments are not satisfactory, because in most cases eggs from the same females gave abnormal results when tested in other ways. The following summarizes the more interesting experiments. Percentages were obtained by counting about 200 eggs.

TABLE II.

	Experiment I.		Experiment II.		Experiment III.		Experiment IV.		Experiment V.	
	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.	Per Cent. Cleavages.	Per Cent. Normal Blastulæ.
Sperm control			23.8		46.6					
Fertilizin control (unradiated)	30.2	0	9.5	0	5.2	1	20.2	.5	15.5	
Fertilizin 2 min. radiation.....	24.3	few	14.1	1	10.8	3	13.5	0	28.5	2
Fertilizin 5 min. radiation	21.6	"	17.9	0	8.4	0	10.5	0	87.2	
Fertilizin 15 min. radiation ...	17.5	0	15.6	.5	9.5	0	13.6	0	52.1	

Since the effect of x-radiation on fertilizin seems to be similar to its effect on enzymes, it is of interest to note the fact that the

efficiency of the agglutinin contained in fertilizin, like pepsin (Euler, p. 132) varies with the square root of the concentration. If the efficiency is measured by the number of seconds the sperm remain agglutinated, and the concentration is measured by units of strength, the curves in Figs. 1, 2, and 3 are obtained for the readings of July 14, August 10, and August 11, respectively. The average is shown in the dotted line of Fig. 4. If an equation is worked out for this curve, we obtain $y^2 = 11x$ where y represents the efficiency and x the concentration. This equation is plotted as a solid line on Fig. 4. In the higher dilutions, of which a greater number of values were averaged, and where readings could be made more accurately, the curves coincide very closely. In the less dilute portion the coincidence is not so marked, but is still within the limits of experimental error.

The writers are not now able to offer an opinion as to whether or not fertilizin has the character of an enzyme. The coincidence, however, in the behavior of this substance, when treated by x-rays, to that of true enzymes, is indeed striking.

While the nature and composition of fertilizin are as yet unknown, it is a cell-extractive which is capable of undergoing changes under the action of experimental agents such as radiation by x-rays. Possibly it, or its forerunner, exists in the egg in combination. Among the other constituents of *Arbacia* eggs, this substance stands as one which, at least in solution in sea water, is able to bring about certain reactions on the part of sperm, and these reactions are subject to experimental modification. This justifies the inference that this substance or perhaps some similar one within the egg may be capable of undergoing modification in its relations to the various intra-cellular activities.

In this modification we may look for the seat of part of the changes which are brought about in living tissues and especially egg cells by radiation. The Hertwigs, Packard and others have shown that the chromatin of such cells is affected, and there is good evidence that the cytoplasm as well is influenced. Changes in their activity have also been demonstrated in the case of enzymes. These experiments add still another to the list of substances which are affected by the action of x-rays. It is

probable that fertilizin is simply one example of a group of substances which may be the object of such action (but an example which may be studied). It is to be noted that these experiments render untenable the conclusion of the Hertwigs, that chromatin is the chief and perhaps exclusive seat of the effects of radiation upon eggs. Fertilizin is a substance doubtless without morphological representation in the structure of the egg; yet it may suffer considerable modification from x-ray treatment.

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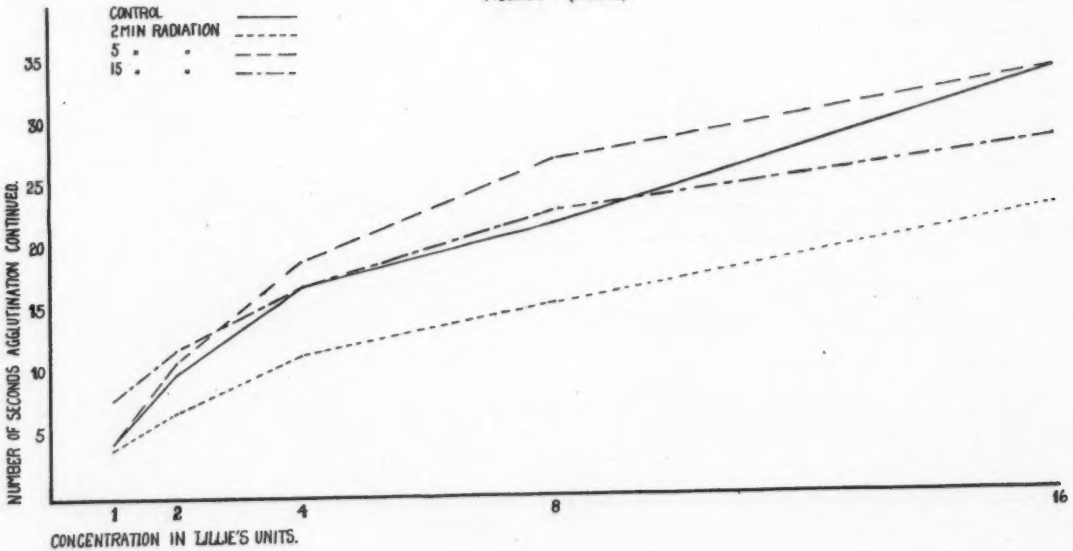
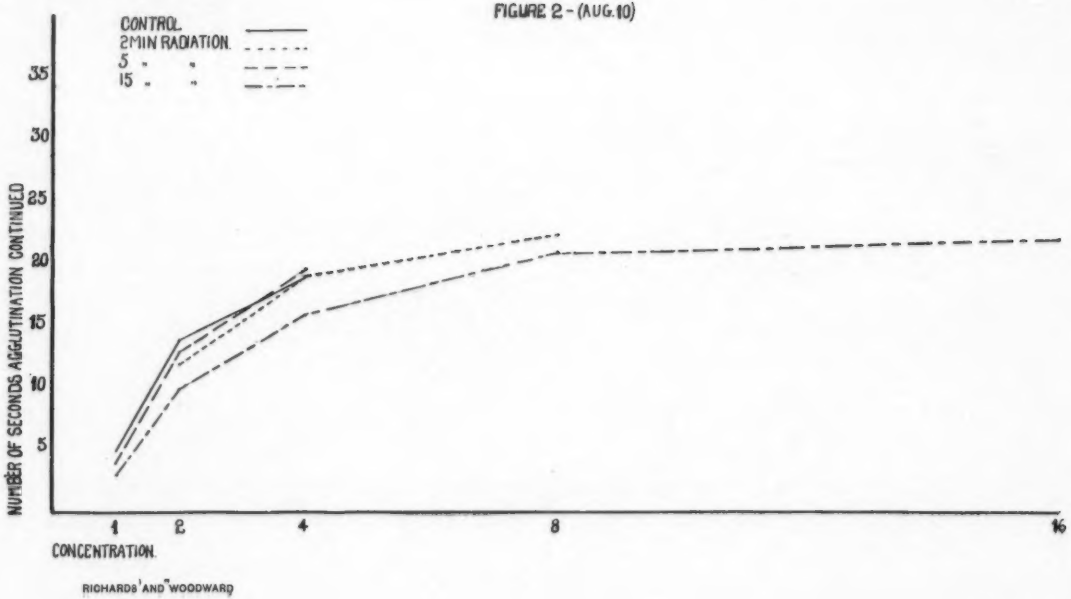
FIGURE 1 – (JULY 14)



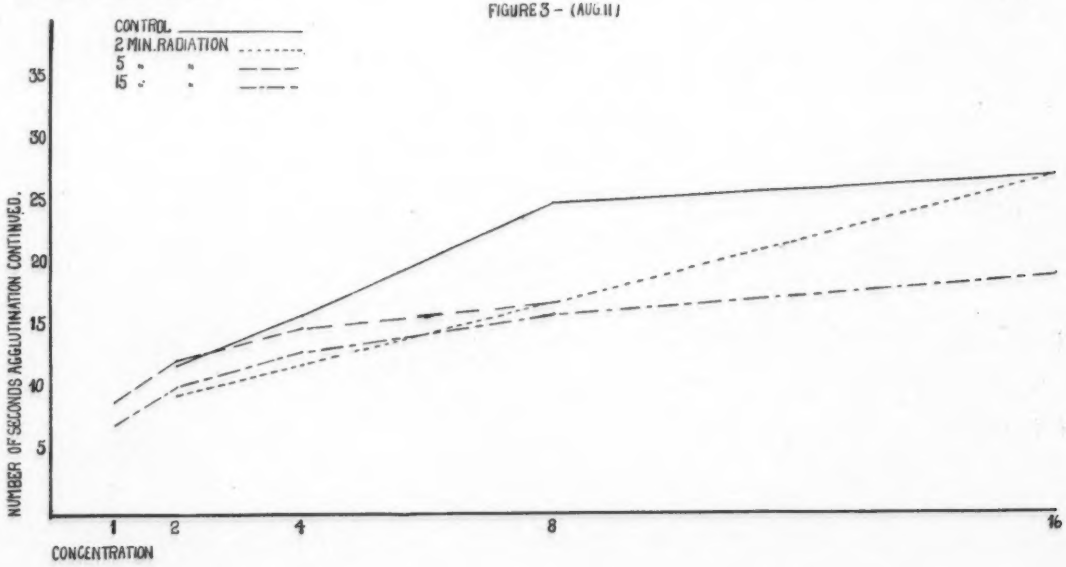
RICHARDS AND WOODWARD

FIGURE 2 - (AUG. 10)



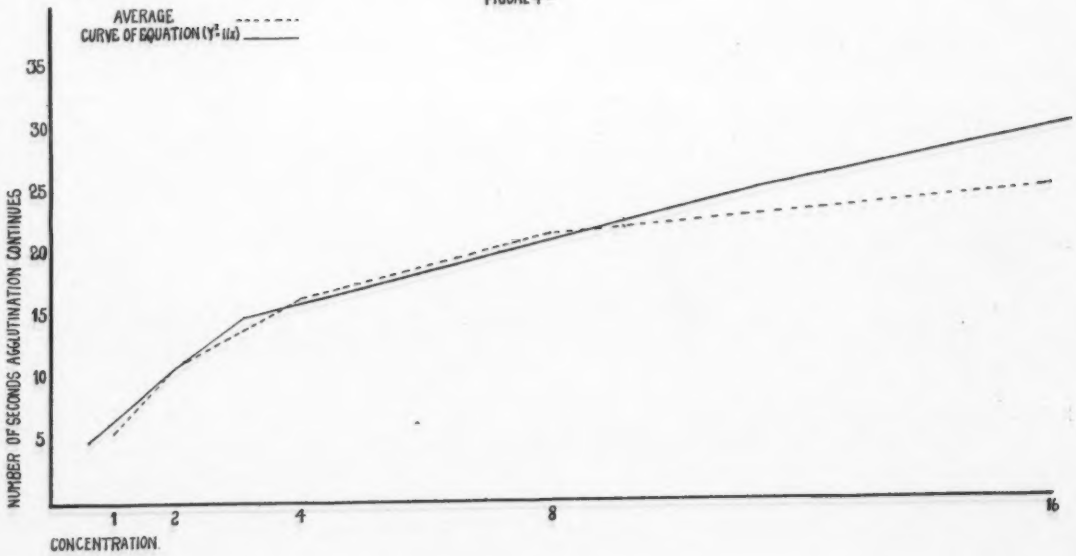
RICHARDS AND WOODWARD

FIGURE 3 – (AUG. 11)



RICHARDS AND WOODWARD

FIGURE 4 –



RICHARDS AND WOODWARD

CAN A SINGLE SPERMATOZOÖN INITIATE DEVELOPMENT IN ARBACIA?[1]

OTTO GLASER.

During the summer of 1913 while making the camera lucida tracings on which I have based my comparisons between the volumes of the unfertilized and fertilized ova of *Arbacia*,[2] it became necessary, in order to prevent rotation on the part of the eggs, and the consequent necessity of readjusting the focus, to employ very attenuated suspensions of sperm. The result of the highest dilutions used in these experiments, however, gave an unforeseen result since the appearance of the fertilization membranes was either very much delayed, or failed entirely to take place. This observation suggested the idea of a mass effect of the spermatozoa, and the possibility that this might play a rôle in normal fertilization.

At that time I had already made observations which had convinced me that the fertilization membrane in this egg is not formed de novo, but is preformed in the unfertilized egg, and simply rendered visible by changes occurring at the time of impregnation.[3]

The mechanism through which the fertilization membrane becomes visible will be dealt with in detail at another time; for the present it is sufficient to say that the absorption of water plays an important rôle. It occurred to me therefore that the prevention of this absorption and perhaps the prevention of fertilization itself might be possible even with the employment of more concentrated suspensions of sperm, if the eggs were first treated with Ca. As a matter of fact, it was either difficult or impossible to fertilize eggs so treated. The spermatozoa were active enough, but failed to enter, and fertilization membranes did not appear The following protocol is typical: In a small watch

[1] From the Marine Biological Laboratory at Woods Hole, and the Zoölogical Laboratory of the University of Michigan.

[2] "The Change in Volume of *Arbacia* and *Asterias* Eggs at Fertilization," BIOLOGICAL BULLETIN, Vol. XXVI, pp. 84–91.

[3] "On Inducing Development in the Sea-Urchin (*Arbacia punctulata*), together with Considerations on the Initiatory Effect of Fertilization," *Science*, Vol. XXXVIII., pp. 446–450.

crystal, 4 volumes of fairly dense egg-suspension in sea-water+2 volumes n $CaCl_2$. After two minutes washed in sea-water.

12.26 insemination moderate.
12.28 0 fertilization membrane.
12.30 1 " "
12.34 1 " "

Control normal. All eggs with fertilization membranes in 3–5 minutes after insemination. 100 per cent. cleavage. Hundreds of eggs examined in both control and experiment. In Ca-eggs very few divisions.

In connection with these experiments I noticed that insemination with great excesses of sperm frequently led to results at variance with the above, for fertilization membranes appeared about the majority of the eggs despite the use of Ca, and these eggs developed. This experience strengthened my belief, not only in the validity of the Ca-experiments, but also in the correctness of the original idea, namely that the number of spermatozoa that come into contact with the egg may make a difference.

Encouraged by this result, I diluted a sperm-suspension until only the faintest trace of opalescence remained. Several drops of this attenuated fluid were then drawn up into a medicine dropper of medium size and expelled quantitatively. If the dropper, which of course remained infected with sperm, was then used to agitate eggs in a small quantity of sea-water by carefully drawing the water in and expelling it several times, it was found that very soon a few spermatozoa had attached themselves to every egg. In an optical diameter, 4 to 5 sperm could easily be distinguished, but I awaited further changes in vain, despite the fact that the spermatozoa seemed to have reached the eggs, exhibited the usual amount of activity, and were potent in 100 per cent. of the cases when applied in larger quantities to eggs of the same lot. The following experiment is illustrative:

12.17 insemination with infected pipette.
12.18 0 fertilization membranes.
12.19 0 " "
12.20 1 " "
12.21 2 " "
12.22 3 " "
12.30 3 " "

Control normal; all eggs with fertilization membranes in 3 to 5 minutes. Experimental eggs examined at irregular intervals throughout the day, but no increase in the number of membranes.

Whether the appearance of a fertilization membrane, and impregnation itself will fail to take place in other eggs under similar conditions cannot be predicted, and is perhaps even improbable. With the eggs of *Arbacia punctulata* however I repeated these tests so often that I cannot doubt the correctness of my observations, and I therefore fail to understand Kite's[1] claim that he succeeded in calling forth a fertilization membrane in this egg by means of a single spermatozoön. I imagine that his method involved factors whose importance was unsuspected, since he says: "The real difficulty with this type of experiment is not the size of the spermatozoön, but the fact that when four or five are injected into the egg-jelly, they usually swim out and away from the egg. This necessitates the making of many injections in order to get a single spermatozoön to attach itself to the vitelline membrane and start the reaction." The "making of many injections" very likely involves touching the vitelline membrane an equal number of times, which recalls an experiment mentioned in my earlier paper[2] in which fertilization membranes were induced by surrounding the eggs with large numbers of minute infusoria. Observation indicated a continuous bombardment of the ova.

A quantitative relation between the rate of appearance of the membrane and the agencies, spermatozoa, normally calling it forth is really no more surprising than the efficacy of Ca as an inhibitor. Since now sea-water of sufficient hypotonicity will of itself call forth membranes[3] one may expect the exact reverse of the Ca-experiments if one immerses the eggs briefly in hypotonic solutions. Such ova, if not submerged too long so that the

[1] G. L. Kite, "The Nature of the Fertilization Membrane of the Egg of the Sea Urchin (*Arbacia punctulata*)," *Science*, Vol. XXXVI., pp. 562–564.

[2] *Science, loc. cit.*

[3] In my preliminary communication (*Science, loc. cit.*) I considered the method of "inducing" a fertilization membrane in *Arbacia* by means of hypotonic sea-water new. Schücking however described this procedure in the year 1903. (*Arch. f. d. ges. Physiol.*, Vol. 97, p. 85.) The same method was used on *Arbacia* eggs by McClendon in 1910. (*American Journ. Physiol.*, p. 246.)

appearance of the membrane would have to be attributed to the hypotonic treatment itself, should be capable of fertilization by means of the sperm-infected medicine dropper. Actually under these circumstances fertilization with only 4 to 5 spermatozoa visible in the optical equator is possible in a considerable number of eggs.

PROTOCOL.

In a watch crystal 3 volumes of sea-water+3 volumes of distilled. Added 1 volume of an egg-suspension in normal sea-water. At the instant when the first indications of membrane "initiation" were noticeable added 3 volumes of "double sea-water," *i. e.*, sea-water whose volume had been reduced one-half by boiling. By means of a sperm-infected pipette every egg was provided with 4 to 5 spermatozoa. In a series of microscopic fields the number of undivided eggs was later compared with the number that had divided. The results were:

Experiment I.		Experiment II.	
Undivided.	Divided.	Undivided	Divided.
7	2	4	1
4	3	3	2
4	4	12	2
7	2	18	0
1	2	6	5
6	0	6	0
2	4	7	4
5	1	8	0
5	4	8	5
5	2	14	4
3	2	14	3
5	3	12	3
5	3	13	1
6	5	8	2
2	6	6	3
4	3	7	1
		13	2
		6	1
		6	3
		10	1
		8	2
Total...71	46	189	45
Per cent.61	39	81	19

Controls: Normal eggs+usual amount of sperm = 100% Fertilization. Eggs treated as above+usual amount of sperm = 100% Fertilization. This is in sharp contrast with the earlier experiments in which the operations were carried out at the same

dilutions but without the brief fore-treatment with hypotonic sea-water. Results which harmonize with these but prove less satisfactory on account of injuries to the eggs can be gotten by the use of heat. In this case one might think of a parthenogenetic effect, but in *Arbacia* at least, it is not easy to confuse the usual parthenogenetic cleavage with normal two or four-cell stages.

It is very easy to misunderstand these experiments and to draw wrong conclusions. There is no more doubt in *Arbacia punctulata* than in any other form that a single spermatozoön is sufficient to carry out the biparental effect. Furthermore the experiments with dilute sperm do not in anyway enable us to prejudge what would happen in another egg under similar conditions nor do they warrant the inference that the initiation of development by a single sperm is impossible in *Arbacia* ova deprived of their superficial coverings. I feel very sure of this however: In *Arbacia* the appearance of the fertilization membrane after insemination is a sign that the egg investments have allowed the sperm to pass through. This passage has been possible because the coverings have changed. The change depends on a synchronous softening and absorption of water, the latter having consequences as the result of which the membrane becomes visible. Inasmuch as the becoming visible of the membrane is a reliable index of fertilization, and one of the consequences of fertilization is the division of the ovum, we may say that the initiation of development by a single spermatozoön in this case is impossible because a single sperm cannot effect those changes in the egg-coverings which will permit it to reach the protoplasmic surface film that lies beneath. The situation is exactly as though the entrance to a room were blocked by a barrier which a single man could not break down, although a group of ten might. Once broken down, any one of the men could cross the threshold, but for the opportunity of doing this, the services of the others would be needed. With this analogy in mind, the statement that a single spermatozoön cannot except possibly under special conditions, fertilize the normally invested egg of *Arbacia punctulata*, would appear to agree with the facts.

ZOÖLOGICAL LABORATORY,
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November 12, 1914.

STUDIES ON THE PHYSIOLOGY OF REPRODUCTION IN THE DOMESTIC FOWL. XII.

On an Abnormality of the Oviduct and Its Effect upon Reproduction.[1]

MAYNIE R. CURTIS.

In a recent paper Pearl and Curtis (1914) have shown that when the passage of an egg through the oviduct is prevented by surgical interference with the duct the sex organs pass through their normal reproductive cycles. The oviduct functions to the level where the passage is interrupted and the egg is then returned into the body cavity. The eggs thus set free may be absorbed without causing any serious disturbance in metabolism. In a paper still in press (Curtis and Pearl) it has also been shown that congenital or acquired obstructions to the oviduct may occur without artificial interference and that the results in such cases are the same as in the former cases.

The following case was recently brought to our attention by Mr. J. C. Hawkes, Poultryman at the Maine Agricultural College poultry plant.

A year and a half old Rhode Island Red bird was killed for meat. She was well grown, in good flesh and in every respect was perfectly normal in appearance. When an incision was made to remove the viscera a full sized membrane shelled egg slipped into the opening. Mr. Hawkes then kindly turned the bird over to us for examination.

The eggs and egg membranes shown in Fig. 1 were all removed from the body cavity of this bird. These represented every possible stage of absorption of the egg from a normal membrane shelled fresh egg to the collapsed empty membranes shown in the fourth line of the figure. Some of the eggs and some of the empty membranes were free in the body cavity. Some were

[1] Papers from the Biological Laboratory of the Maine Agricultural Experiment Station No. 76.

partly or entirely enclosed by peritoneum. In several instances two eggs or an egg and a bunch of membranes were walled off together. The last line of the figure shows collections of empty membranes enclosed in peritoneum. These peritoneal covered masses were attached by suspending strings or folds of peritoneum. The large mass at the right end of this line contains a very large number of these empty membranes. A larger view of it is shown in Fig. 2. The second line from the bottom of Fig. 1 shows collapsed empty egg membranes of which some are single and some two or three tightly packed together. The three top lines of the figure show eggs in various stages of resorption. One was a normal fresh egg in a single egg membrane. Ten had evidently been normal eggs but at the time of autopsy they contained a homogeneous mixture of yolk and albumen which had lost the gelatinous character of fresh egg albumen. Each of these eggs was enclosed in a single egg membrane. The other four eggs were double eggs. These eggs were much like the double eggs (*ovum in ovo*) described by Parker (1906), Patterson (1911) and by many other writers. (The appended bibliography is supplementary to the one given by Parker 1906.)

The eggs of this sort described in the literature had all been laid. Most of them have had shell on one or both of the concentric components. The double eggs found in the body cavity of this Rhode Island Red hen had no shell on either the enclosed or enclosing egg. The nature of the contents of the double eggs differed in each of the four cases. In one both enclosed and enclosing egg contained yolk. The yolk and albumen of the enclosing egg were somewhat mixed, although they did not yet constitute a homogeneous fluid. In fact the currents or streams of yolk could be seen in the clear albumen through the semitransparent egg membrane. The yolk and albumen of the enclosed egg were still more distinct although the yolk membrane had already ruptured. The enclosed egg was about the size of the normal egg and the enclosing egg (the third egg in the top line) was the largest egg found in the body cavity. A second double egg was composed of a normal sized enclosed egg which had apparently contained the normal egg parts. The contents had, however, been reduced to a homogeneous brownish-yellow liquid

much thinner than fresh egg albumen. The enclosing egg was only slightly larger than the egg it enclosed and it seemed probable that a second egg membrane had been received directly around the first on its passage back up the duct. A third of the double eggs had two closely applied egg membranes as in the preceding case but the enclosed egg was itself a double egg. The inner egg in this series was a small "witch" or "cock" egg containing a little yolk not enclosed in yolk membrane and a small amount of normal fresh albumen. The outer egg contained only normal fresh albumen. The other double egg was even more remarkable in character as it consisted of a concentric series of four enclosed eggs. The inner one, like the inner egg just described, contained a little free yolk enclosed in normal albumen. Each of the successive enclosing eggs contained only normal albumen. This whole egg was not larger than a normal hen's egg.

These peculiar double formations indicate that an egg did not always pass up the duct in time to get out of the way of a succeeding egg. In case an egg met another yolk it might become enclosed in a double egg or it might change the direction of the incoming yolk If the yolk was ruptured and a part remained in the duct it might furnish the nucleus for a "cock" egg which might then become enclosed in a succeeding egg. Apparently the direction of peristaltic movements became at times much disturbed, as the last double egg described must have passed up and down the duct several times before it was finally extruded into the body cavity.

The visceral organs of the bird were in normal condition. There was a little slightly oily yellowish serous fluid bathing the viscera. The peritoneum was very slightly thickened but otherwise normal. The ovary was normal with a normal series of enlarging yolks and resorbing follicles. It was apparent that the bird was in the midst of a normal reproductive period and was backing membrane shelled eggs into the body cavity and resorbing them with great rapidity.

The oviduct (Fig. 3) was perfectly normal from the funnel mouth to the posterior end of the isthmus. Here the tube abruptly ended blindly at *D*. There was no shell gland or vagina.

The oviduct ligaments were continuous to the posterior end of the body cavity. That is the tube ended in the fold of enclosing peritoneum while the fold continued to the posterior end of the body cavity. The heavy bands of smooth muscle in the ventral ligament (see *E*, Fig. 3) continued to the end of the body cavity —several centimeters beyond the end of the tube. The tube rounded off smoothly at the posterior end and the ligament behind did not present the slightest indication that it had ever contained any oviduct tissue. It seems probable that the duct had never extended any farther than at present. From the embryonic history of the oviduct it is evident that if the actively growing point of a duct should cease at an unusually long distance anterior to the cloaca a blind oviduct of this form might result.

The development of the oviduct according to the account given by Lillie (1908) begins on the fourth day of incubation as a groove-like invagination of a strip of thickened peritoneum on the surface of the Wolffian body or embryonic kidney. The lips of this groove fuse on the fifth day so as to form a short tube open anteriorly to the body cavity and ending blindly posteriorly. The open end of this tube becomes the *ostium tubæ abdominale* or funnel mouth of the oviduct. The posterior end grows backward between the strip of thickened peritoneum and the Wolffian body. It normally reaches the cloaca on the seventh day. The growing point is always a short solid wedge of cells. The duct receives its lumen a short distance anterior to this. On the twelfth day of incubation the primordium of the shell gland is distinctly visible as an expansion of the lower end of this tube.

The most probable explanation of the abnormality of the oviduct found in the case described is that in early embryonic development (probably on the sixth or seventh day of incubation) the backward growth of the primordial oviduct stopped permanently while the differentiation of the part already formed continued in the normal manner.

As in other cases where the passage of the egg is prevented the sex organs passed through their normal reproductive cycles; the oviduct functioned as far as the point where the passage was interrupted; the eggs were then returned to the body cavity and resorbed. The number of eggs and empty egg membranes found

in this fowl which was apparently in a perfectly normal physical condition show that a bird possesses very great power of resorption of its own proteins from the peritoneal cavity. Such resorption does not necessarily cause metabolic disturbances.

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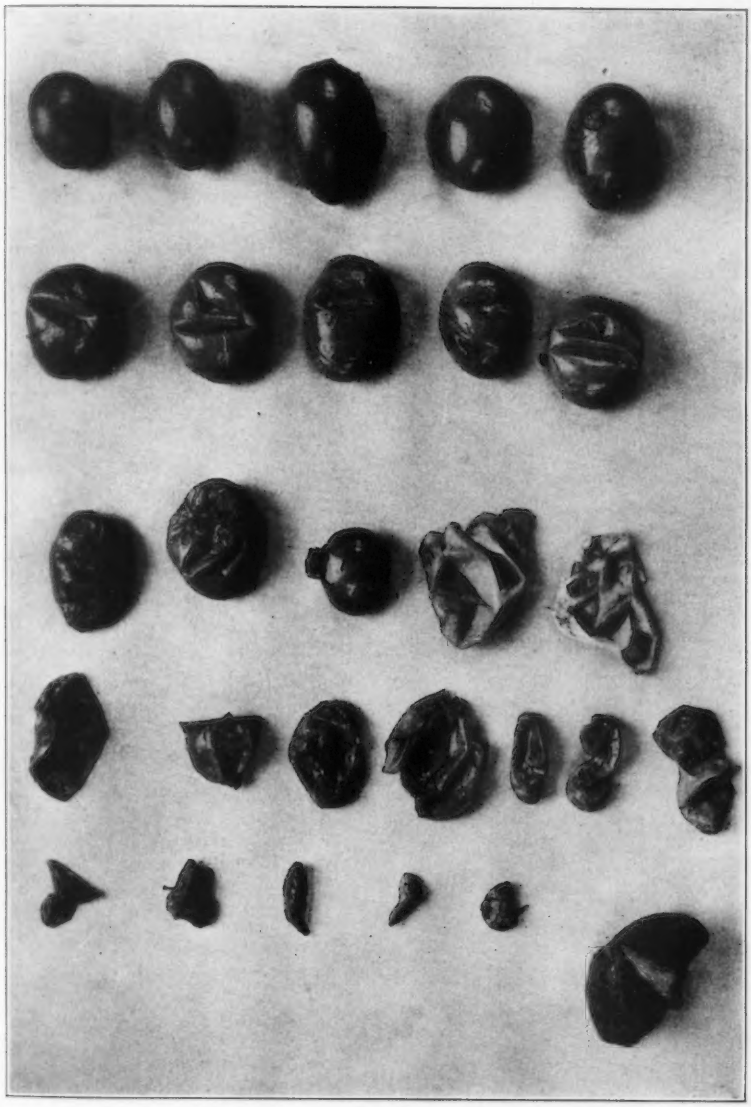
Explanation of Plate I.

Fig. 1. Eggs and egg membranes removed from the body cavity of a Rhode Island Red fowl.



MAYNIE R. CURTIS. FIG. I.

Explanation of Plate II.

Fig. 2. Natural size photograph of the large peritoneal covered mass of egg membranes shown at the lower right hand corner of Fig. 1. This is cut across and opened back to show its composition.

Fig. 3. Photograph (greatly reduced) showing the oviduct of the bird from which the eggs in Fig. 1 were taken. *A* = funnel; *B* = albumen secreting region; *X* = isthmus ring; *C* = isthmus; *D* = blind end of the oviduct; *E* = mass of smooth muscle in ventral ligament posterior to the end of the oviduct.

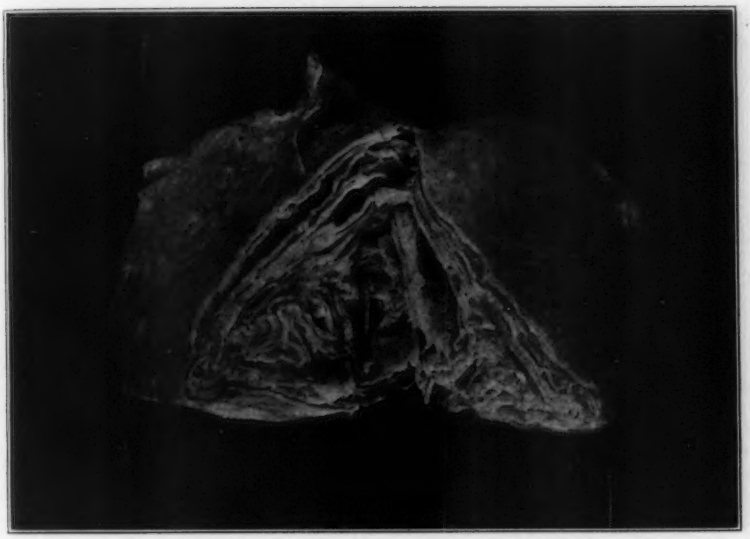
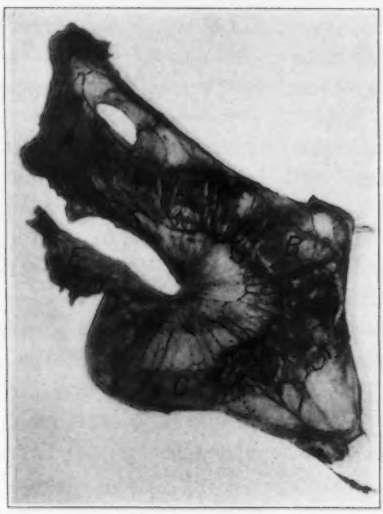


FIG. 2.



MAYNIE R. CURTIS. FIG. 3.